

## THE EFFECT OF AGE ON BODY ENERGY CONTENT OF THE ANNUAL CYPRINODONT FISH, *NOTHOBRANCHIUS GUENTHERI*

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### INTRODUCTION

RECENTLY THERE has been a growing interest in the use of the annual fish, panchax, in experimental aging studies. The relative value of using fish in aging research has been discussed by Woodhead (1978), and various researchers are beginning to explore this approach to gerontological research (e.g., Walford and Liu, 1965; Liu and Walford, 1966, 1969, 1970; Cannon and Davidson, 1978; Markofsky *et al.*, 1972, 1973, 1976, 1979). Of the wide variety of annual fish available, the *Nothobranchius guentheri* (family *Cyprinodontidae*) appears to be the easiest to maintain in the laboratory with a minimum of husbandry problems. This fish is a mud spawner with a normal lifespan in captivity of 12 to 18 months. Their origin is East Africa where they have evolved in an environment having an annual rainy and dry season. Because they cannot normally survive the dry season, their lifespan has been scaled to approximately one year.

In the study reported here the energy content of the complete fish body was measured in a bomb calorimeter and correlated vs. fish age in an attempt to measure collagen crosslinking energetics. However, a slight decrease in body fat content with increasing age (Markofsky, 1976) apparently masked the crosslinking energy storage effects.

### MATERIALS AND METHODS

Male and female *Nothobranchius guentheri* fish populations of various young ages were purchased commercially.\* Several lots of 50 or more were purchased over a period of 2 years at ages between fry and 2 months. These fish were then maintained in laboratory facilities in unheated tanks at a density of approximately 1 fish/1 in 10 and 15 gallon aquaria. Young fish were fed live brine shrimp and then switched to TetraMin<sup>®</sup> from an automatic feeder. Freeze dried brine shrimp were mixed into their diets once or twice a week. The room temperature was maintained between 20 and 23°C during the entire experiment. Groups of 5 males and 5 females were culled from the stock at monthly intervals and dried in a vacuum oven at 40°C for 24 hours. They were then sealed in airtight capsules and stored at room temperature for later bomb calorimetry testing.

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\*All fish in this study were purchased from Mr. Ed Warner, 507 John Street, Rockford, Illinois.

A Parr model 1241 Automatic Adiabatic Bomb Calorimeter equipped with a model 1245 semimicro bomb system was used for testing the dried fish. This system is capable of testing samples from 25 to 200 milligrams liberating energies up to 1200 calories. The dried fish were first pelletized and then burned individually, except for the 1 month old fry which were pelletized in groups of 3 or 4 per test. Calorimeter data were analyzed on a computer using custom written software. The calorimeter system was calibrated with commercially available benzoic acid calorific standards. The bomb constant was determined from the average of 10 benzoic acid calibration tests and the standard deviation of the 10 tests was 17 cal/g.

## RESULTS

Table 1 presents the results of body energy content measurements made with the bomb calorimeter on 43 (20 M, 12 F, 11 unsexed) *Nothobranchius guentheri* of various ages. The energy values are per gram of *dry* whole body weight and they show a significant decrease with increasing age. The larger standard error values for females may be due to variations in egg contents among the females.

## DISCUSSION

It is now clear that in the biological world increasing age produces increasing molecular crosslinking among protein macromolecules (Eyre, 1980; Goosey and Zigler, 1978; Panigrahy and Patnaik, 1976; Deardin and Odusina, 1973; Stenzelet *et al.*, 1974; Sinex, 1964). Walford *et al.* (1969) have shown that the soluble/insoluble (*i.e.*, uncrosslinked/crosslinked) collagen ratio decreases with increasing age in the body of the annual fish *Cynolebias bellottii*. Recently, however, Cannon and Davison (1978) reported the negative results of a similar soluble/insoluble collagen ratio study on the skin of *N. guentheri*.

Since the chemistry of living biological systems obeys the classical laws of chemical reactions, then each biochemical reaction that occurs must be accompanied by a decrease in the (Gibbs) free energy of the system. Consequently, the net effect of biochemical reactions producing molecular crosslinking will be to decrease the free energy of the biological system. Since a bomb calorimeter measures the total molecular energy content of a sample, then all other things remaining equal, the effect of increasing molecular crosslinking with increasing age should appear as a decrease in body energy content per unit weight with increasing age. Bomb calorimetry has been used for some time in the field of synthetic polymers to determine the free energy decrease in polymerization reactions (*e.g.*, see Dainton and Ivin, 1950; Joshi and Zwolinski, 1967; Sawada, 1976). Therefore, it should

TABLE 1. SPECIFIC BODY ENERGY CONTENT vs. age of *N. Guentheri*

Group Age (Months)	Sex (Number)	Mean Energy Value (cal/g)*	± Standard Error** (cal/g)
1.0	mixed (11)	4496	155
2.25	M (5)	4579	81
2.25	F (4)	4473	195
3.0	M (5)	4375	68
4.0	M (1)	4010	—
6.0	M (8)	4047	51
6.0	F (3)	4866	401
11.0	M (1)	3800	—

\*Dry weight.

\*\*Standard Error — (Standard Deviation)/  $\sqrt{n}$ .

be directly applicable to the biological use described here since macromolecular crosslinking is morphologically identical to polymerization.

Let  $\Gamma$  be the total energy decrease (deficit) within a living system over some time span due to molecular crosslinking. Then we can write:

$$\Gamma = \gamma mn \quad (1)$$

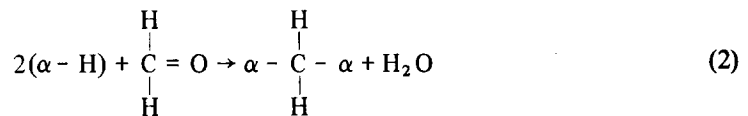
where  $\gamma$  = energy deficit per crosslinked bond,

$m$  = number of crosslinks per macromolecules,

$n$  = total number of crosslinked macromolecules in the system.

Unfortunately, the crosslinking chemistry of protein macromolecules is still largely unknown (Eyre, 1980). Collagen chemistry is currently the most completely understood with typical crosslinks arising from the condensation of aldehydic groups or of an aldehyde and amino group. Collagen is a fibrous protein whose amino acid residues are about 33% glycine, 21% proline and hydroxyproline, and 11% alanine. Collagen is the most abundant protein in living systems, often comprising one-quarter to one-third of the total system protein. Tropocollagen is the basic molecular subunit of collagen. It contains 3 polypeptide chains wound together in a triple helix to form a rigid rod 2800 Å long, 15 Å in diameter, with a total molecular weight of 300,000. The tropocollagen rod structure is stabilized with hydrogen bonding and crosslinking between the component polypeptide chains. Collagen polypeptide chains with no crosslinks are called  $\alpha$  chains. Two  $\alpha$  chains crosslinked together is called  $\beta$  collagen, and when all three chains are crosslinked it is known as  $\gamma$  collagen. Bailey (1970) has studied the nature of the crosslinking in the collagen of various fishes and concludes that collagen crosslinking is fundamental and independent of different morphological fiber arrangements.

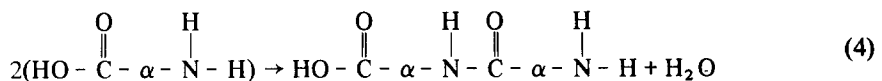
Though a variety of crosslinking agents may be involved in biological aging, for purposes of illustration here we will focus on only two different types. First we consider a simply aldehyde covalent crosslink between two  $\alpha$  collagen polypeptide chains.



In equation (2) we have used formaldehyde to produce a methylene crosslink bridge between two  $\alpha$  chains. A bond energy calculation for this reaction gives a net crosslinking energy deficit of 13 Kcal/mole. Consequently, the energy deficit per crosslink,  $\gamma$ , is given by:

$$\gamma = \frac{13}{A} \text{ Kcal/crosslink} \quad (3)$$

where  $A$  is Avogadro's number ( $6.02 \times 10^{23}$  molecules/mole). A simple peptide bond crosslink can be modeled as follows:



A bond energy calculation for the reaction of equation (4) gives a net energy deficit of 5 kcal/mole, and for this reaction:

$$\gamma = \frac{5}{A} \text{ Kcal/crosslink} \quad (5)$$

For purposes of calculation we will assume an average value of  $\gamma$  of 10/A Kcal/crosslink.

Let  $W$  be the dry weight of the biological system, and assume that it is 80% collagen. Then we can estimate the maximum total number of crosslinked collagen chains in the system,  $n$ , as:

$$n = 0.8 W \left( \frac{A}{M} \right) \text{ chains} \quad (6)$$

where  $A$  = Avogadro's number, and  $M$  is the  $\alpha$  chain molecular weight ( $10^5$ ).

Finally, we must estimate the maximum number of crosslinks possible per  $\alpha$  chain. We assume crosslink sites are readily available along the entire length of the chain. Atkins (1977) has introduced what he calls a "test tube brush" model of a proteoglycanhyaluronic acid complex and proposes that tropocollagen fibrils could be crosslinked with such a proteoglycan matrix. If one considers each glycine within the  $\alpha$  chain as a possible peptide crosslink site, then there are about 330 such sites per  $\alpha$  chain. It does not seem unreasonable to consider as many as 500 possible crosslinking sites of all types on each  $\alpha$  chain which will allow crosslinking both within and between tropocollagen fibrils. Combining  $m = 500$  with equations (5) and (6) into equation (1) gives:

$$\frac{\Gamma}{W} = 40 \text{ cal/(g dry Wt)} \quad (7)$$

Thus we should see a maximum energy deficit in the bomb calorimetry data of about 40 cal/g dry Wt for age induced crosslinking. Table 1 shows a much larger decrease in body energy content with increasing age.

Markofsky (1976) has studied the body composition of *N. guentheri* at various points in their lifespan using both longitudinal and cross sectional observations. His data indicate that the total body water content is a constant fraction (78 to 81%) of the fat-free body weight (FFB) independent of age. The protein fraction of the FFB increases between 3 and 4.4 months, remains constant until 12.4 months, then declines slowly. He found the whole body fat content difficult to measure accurately, but from the cross-sectional observations it appeared to reach a maximum of  $3.38 \pm 0.19\%$  at 4.4 months of age. Then it decreased to  $1.63 \pm 0.46\%$  at 8.9 months, increased to  $2.14 \pm 0.62\%$  at 13.5 months, and then decreased again to  $0.73 \pm 0.17\%$  at 15.3 months. Longitudinal observations were made only for body length and total body weight. Though his cross sectional and longitudinal observations did not always lead to the same conclusions, it seems reasonable to conclude from his data that the body fat component of *N. guentheri* decreases over its lifespan by 1 to 2%. This rather small change in total composition escalates to 5 to 10% of the dry body weight when one assumes a constant 80% body water fraction. Taking the energy value of water-free fat to be 9.5 Kcal/g (Swan, 1974) and assuming the protein composition to be

approximately constant over the lifespan, we find that a loss in water-free fat of 5 to 10% of the dry body weight corresponds to a net loss in body energy storage of 475 to 950 calories per gram of dry body weight. This is approximately the decrease in body energy over the lifespan shown in the bomb calorimeter data of Table 1. Therefore, unless we have underestimated  $\gamma$ ,  $m$  or  $n$  by a factor of 10 to 100, the small change in the fishes' body fat component appears to be the main source for the variation in body energy content with increasing age shown in this Table. Any body energy decrease with increasing age due to macromolecular crosslinking chemistry appears to be masked by a simultaneous decrease in body fat fraction with increasing age.

### SUMMARY

Bomb calorimetry measurements were made on dried whole body samples of *N. guentheri* in an attempt to detect the effect of age induced protein crosslinking. The results show a clear decrease in body energy content with increasing age. However, this decrease was larger than expected and was probably due to a slight decrease in body fat component with increasing age rather than crosslinking alone.

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